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Immunization with α-synuclein/Grp94 reshapes peripheral immunity and suppresses microgliosis in a chronic parkinsonism model

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Main points

α-synuclein/Grp94 vaccination, either by splenocytes adoptive transfer or by direct immunization, supports a unique Th1/immune-regulatory mixed profile leading to the first report of suppressed chronic microgliosis in a mouse model of parkinsonism.

Keywords

Parkinson's Disease

misfolding protein

heat-shock protein

immune response

microglia

Abstract

Neuroinflammation mediated by chronically activated microglia, largely caused by abnormal accumulation of misfolded α-synuclein (αSyn) protein, is known to contribute to the pathophysiology of Parkinson's disease (PD). In this work, based on the immunomodulatory activities displayed by particular heat-shock proteins (HSPs), we tested a novel vaccination strategy that used a combination of αSyn and Grp94 (HSPC4 or Gp96) chaperone and a murine PD model. We used two different procedures, first, the adoptive transfer of splenocytes from αSyn/Grp94-immunized mice to recipient animals, and second, direct immunization with αSyn/Grp94, to study the effects in a chronic mouse MPTP-model of parkinsonism. We found that both approaches promoted a distinct profile in the peripheral system -supported by humoral and cellular immunity- consisting of a Th1-shifted αSyn-specific response accompanied by an immune-regulatory/Th2-skewed general phenotype. Remarkably, this mixed profile sustained by αSyn/Grp94 immunization unexpectedly led to strong suppression of microglial activation in the *substantia nigra* and *striatum*, pointing to a newly described beneficial effect of anti-αSyn Th1-responses in the context of PD. This strategy is the first to target αSyn and report the suppression of PD-associated microgliosis. Overall, we show that the αSyn/Grp94 combination supports a distinct and long-lasting immune profile in the peripheral system, which has an impact at the CNS level by suppressing chronic microglial activation in an MPTP model of PD. Furthermore, our study demonstrates that reshaping peripheral immunity by vaccination with appropriate misfolding protein/HSP combinations could be highly beneficial as a treatment for neurodegenerative misfolding diseases.

Introduction

Parkinson's disease (PD), which is by far the most frequent motor-related neurodegenerative pathology (Poewe et al., 2017), falls within the group of 'protein misfolding diseases' together with Alzheimer's disease, amyotrophic lateral sclerosis and other related neurodegenerative pathologies. In these disorders, specific soluble and functional proteins misfold and form oligomeric species that ultimately convert into highly organized fibrillar structures, accumulating within cells or in the extracellular space (Chiti and Dobson, 2017). PD is characterized pathologically by the presence of intraneuronal deposits largely composed of aggregated α-synuclein (αSyn) and by the progressive loss of dopaminergic neurons mostly in the *substantia nigra pars compacta* (SNpc) of the brain (Spillantini et al., 1997; Moore et al., 2005). Even though substantial progress has been achieved in our understanding of different pathophysiological mechanisms involved in PD, its primary cause is yet to be determined and the disease remains incurable.

Another hallmark of PD-affected brains is the presence of robust microglia activation within damaged areas and a state of chronic neuroinflammation (McGeer et al., 1988; Appel et al., 2010). Based on accumulating evidence, it is currently thought that this neuroinflammatory process directly contributes to neurodegeneration and neuronal death (Glass et al., 2010; Virgone-Carlotta et al., 2013; Fakhoury, 2016; Ransohoff, 2016), supporting the notion of non cell-autonomous processes affecting neurodegeneration and a prominent role of glial-mediated immunity during the course of disease.

More recently, a variety of studies have reported alterations of adaptive immunity in PD, including abnormal peripheral $CD4^+$ T-cell pool, higher levels of disease-specific αSyn antibodies in serum, and T cell infiltration into the central nervous system (CNS)

(Brochard et al., 2009; Fakhoury, 2016), and a role for MHC-II-mediated responses within the CNS associated to PD pathology has also been demonstrated (McGeer et al., 1988; Martin et al., 2016). Even though the main components and mechanisms remain to be elucidated, a number of findings in animal models of PD point to a direct role of pathology-associated αSyn species on immune imbalance and disease progression (Allen Reish and Standaert, 2015). In the light of these findings, several immunization strategies have been tested over the past decade (Olson and Gendelman, 2016; Schneeberger et al., 2016; Valera et al., 2016). Although these studies have provided valuable information on the immune bases of PD pathophysiology, it is not yet clear whether specific immunological responses promote or hinder the pathological process in the different stages, and which immune profiles supported by immunotherapy would be ultimately protective in the context of disease. Importantly, no immunization study has so far been reported to both target αSyn and be able to counteract the microgliosis state associated to PD. It is therefore important that novel vaccination strategies with disease-modifying potential, and particularly those with the ability to target αSyn-triggered neuroinflammation, are explored.

In addition to their classical chaperone functions, heat-shock proteins (HSPs) have been more recently found to display diverse immunological activities and are currently thought to play a role in immunity (Binder, 2014). Previously, we found that specific HSPs can significantly modulate the αSyn-elicited peripheral immunity *in vitro* and in immunized mice in the absence of pathology (Labrador-Garrido et al., 2014; Labrador-Garrido et al., 2016).

The goal of the present proof-of-concept study has been to examine the effects, in the context of PD, of αSyn/HSP vaccination on the peripheral system and its possible impact on neuroinflammation, particularly on microgliosis. We report that vaccination

with αSyn/Grp94 leads to a long-term reshaping of peripheral immunity, characterized by a distinct and newly reported Th1/immune-regulatory decoupled profile at the end-stage of chronic PD, and involving both cellular and humoral arms of the immune system. Remarkably, this immunotherapeutic strategy is, to the best of our knowledge, the first to target αSyn and to suppress the development of PD-associated microgliosis.

Materials & Methods

Animal care, vaccination and MPTP treatment

C57BL/6N male mice (Charles River Laboratories, Barcelona, Spain and the University of Seville Center for Animal Production and Experimentation, Seville, Spain), aged 2-3 months, were housed in a regulated temperature environment (22±1 ºC) on a 12 h light/dark cycle, with *ad libitum* access to food and water. Mice were kept for 1 week in the local animal house before the start of the immunization protocol, to allow them to acclimatize to their new environment. Animals from each experimental group were allocated to different cages such that each cage contained up to 5 mice, in every case corresponding to a mix from different experimental groups.

Mice were vaccinated either by adoptive transfer (AT) of splenocytes isolated from immunized donor mice or by direct immunization (DI), as described below in this section. Seven or 14 days after the end of AT or DI vaccination protocol, respectively, mice were rendered parkinsonian by the chronic administration of 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) at a dosage of 20 mg/kg s.c., given 3 times a week for 3 months (Sigma, St Louis, MO, USA) as previously described (Munoz-Manchado et al., 2016). The healthy control animals (saline) received similar treatment with saline solution alone (0.9% NaCl; Sigma).

The animals were sacrificed one month after the end of the MPTP treatment, under deep anesthesia induced by a combination of 100 mg/kg ketamine (Pfizer, New York, NY, USA) and 10 mg/kg xylazine (Bayer, Leverkusen, Germany). All experiments were performed according to the European Directive 2010/63/EU and the Spanish RD/53/2013 for the protection of animals used for scientific purposes. The study was approved by the Animal Research Committee of the University Hospital Virgen del Rocío (University of Seville) and the CABIMER Animal Research Committee (CEEA-4/2014). After sacrifice, the spleen and 500–700 μL of blood were extracted for analysis.

Proteins

Full-length human Wt αSyn protein was over-expressed in *Escherichia coli* BL21 (DE3) cells using the pT7-7 plasmid and purified as described previously (Roodveldt et al., 2013). The purity and monomeric state of the αSyn preparation (>95%) was assessed by 15% SDS-PAGE, mass spectrometric analysis, and 4–10% native PAGE (Lonza, Basel, Switzerland), as previously described (Roodveldt et al., 2013). To remove any possible content of endotoxin or αSyn aggregates, the αSyn solutions were passed through a 100 kDa cutoff Amicon filter (Millipore, Billerica, MA, USA), before being stored at -80°C. The concentration of αSyn was determined by means of a Micro BCA Reagent kit (Pierce, Rockford, IL, USA). Endotoxin levels in the protein preparations were measured by the ToxiSensor Chromogenic LAL Assay kit (GenScript, Piscataway, NJ, USA), and the values obtained were <1 endotoxin unit (EU)/mg protein. Highly purified, endoxotin-free recombinant HSPC4 (also known as Grp94 or Gp96) protein was purchased from Abcam (cat. No. ab92290).

Immunization protocol

The same immunization protocol was used for immunizing donor mice for the AT and the DI approaches. Pre-incubation mixtures were prepared with αSyn and Grp94 proteins at a 1:1 molar ratio (αSyn:Grp94) or with αSyn or Grp94 proteins separately, in 20 mM HEPES (pH 7.5) for 2 h at 42 °C (Rosser et al., 2004). Subsequently, the pre-incubated mixtures were diluted in PBS (approximately a 14-fold dilution of the pre-incubation mixtures) before use in the immunization protocols. For mouse immunizations, animals were injected s.c. on d0 with the pre-incubated mixtures, namely αSyn/Grp94 (1.28 μg/8 μg), αSyn (1.28 μg, αSyn group), Grp94 (8 μg, Grp94 group), or buffer (vehicle group), in 300 μL of CFA (Complete Freund's Adjuvant, Difco, BD Biosciences) delivered in three injections: one in the cervical region and two in the lumbar region, one above each hindlimb. The same procedure was repeated on d7 and d28 with IFA (Incomplete Freund's Adjuvant, Sigma-Aldrich).

The protocol carried out for the adoptive transfer of murine splenocytes (AT) procedure was the following: one week after the second boost (d35), donor mice were sacrificed, and the spleen and 500–700 μL of blood were extracted for analysis. A pool of splenocytes isolated from 5 immunized mice was prepared by mixing $2x10^7$ cells per mouse from every experimental group. On d0, each mouse was injected intraperitoneally with 10^7 cells; 5 recipient mice per group were used (N=5). Seven days later, acceptor mice were rendered parkinsonian by MPTP treatment as described above.

Histological analyses

After transcardial perfusion at r.t. with 50 mL of PBS (Sigma-Aldrich) and subsequently with 50 ml of 4% paraformaldehyde (Sigma-Aldrich) in PBS, the brains were immediately removed and fixed overnight at 4 ºC with 4% paraformaldehyde in

PBS. After fixation, the brains were cryoprotected on 30% sucrose (Sigma) in PBS and included on OCT (Tissue-Tek Sakura, Tokyo, Japan), and coronal sections (thickness 30 μm) were cut on a cryostat (Leica, Wetzlar, Germany). Immunohistological detection of ionized calcium-binding adapter molecule 1 (Iba1) and glial fibrillary acid protein (GFAP) was performed as previously described (Munoz-Manchado et al., 2013) by using, respectively, polyclonal anti-Iba1 (1:500; Wako Chemicals, Tokyo, Japan) and polyclonal anti-GFAP (1:500; Dako, Carpinteria, CA, USA) antibodies, and a secondary peroxidase-conjugated antibody kit (Dako). Images were obtained using a light-transmitted microscope (Olympus BX61; Olympus, Tokyo, Japan) and a refrigerated digital camera (Olympus DP70; Olympus).

Stereology and densitometry

Unbiased stereological analysis was performed by systematic random sampling using the optical dissector method (West, 1993). Quantification of $Iba1^+$ cells in the SNpc was carried out in the region spanning -2.92 mm to -3.40 mm relative to Bregma according to the mouse brain stereotaxic atlas (Paxinos and Franklin, 2004). Only cells lateral to the medial terminal nucleus of the accessory optic tract were quantified in order to have a clear separation from the adjacent ventral tegmental area (Sauer et al., 1995; Munoz-Manchado et al., 2013; Munoz-Manchado et al., 2016). Systematic random sampling analysis of striatal $Iba1^+$ cells was performed from a total of six slices covering the total rostro-caudal extent of the striatum [from 1.54 mm to -0.10 mm relative to Bregma, (Paxinos and Franklin 2004)]. Reference volumes for each section were outlined under low magnification (4x). The numbers of total, resting and activated $Iba1^+$ microglial cells were counted at high magnification (40x) using a 12724.7 μm^2 x 20 μm optical dissector, with a guard volume of 5 μm to exclude any possible artifact on the cut

surface of the sections. To avoid any bias in the measurement of microglial activation, $Iba1^+$ cells were counted only if they had a cell body volume greater than 300 μm^3 [measured by an optical rotator; (Tandrup et al., 1997)]. All stereological procedures were performed using the New CAST™ system (Visiopharm, Hørsholm, Denmark) with a coefficient of error (CE) ≤0.09. In all cases, the CE was lower than 50% of the observed group variance (5-15%), indicating a high level of accuracy in the stereological analysis (Gundersen et al., 1999).

The optical density (OD) of SNpc $GFAP^+$ staining, used to estimate astrogliosis, was obtained from a total of six slices from each animal, covering the same region of the SNpc analyzed by unbiased stereology. OD was measured from digitized pictures using the NIH Image software (ImageJ; NIH, Bethesda, MD, USA) as previously described (Munoz-Manchado et al., 2016).

Isolation of murine splenocytes

Splenocytes were isolated from excised mouse spleens by perfusion with 10 mL of PBS, after which the erythrocytes were lysed by osmotic shock. The number of cells was determined by hemocytometer counting.

Determination of $CD4^+$, regulatory T cell (Treg) in isolated splenocytes

After isolation of splenocytes as described above, 10^6 cells were labeled with anti–CD4-FITC, anti–CD25-APC, and anti–Foxp3-PE antibodies (BD Biosciences, cat. No. 553047, 561048 and 560408, respectively), by following the manufacturer's instructions. Flow cytometry analysis was performed with a FACS Calibur cytometer using CellQuest Pro (BD Biosciences) software. The regulatory T (T_{reg}) cell population

was calculated as the percentage of cells positive for CD4, CD25, and Foxp3 staining ($CD4^+CD25^+Foxp3^+$) among the $CD4^+$ lymphocyte population.

***Ex vivo* stimulation of splenocytes**

Splenocytes were isolated from the spleen of immunized mice after sacrifice, as described above. 3 x 10^6 cells from each mouse were divided and cultured in 3 wells in RPMI medium (BioWhittaker, Verviers, Belgium) containing 10% inactivated foetal bovine serum (iFBS). Each well was treated with medium alone (Ctrl), with αSyn (20 μg/mL) or with a combination of anti-CD3/anti-CD28 (following the manufacturer instructions (cat. No. 16-0031-85 and 16-0281-82, respectively; eBioscience, San Diego, CA, USA). After incubation for 24 h, cell cultures were treated with GolgiStop™ (BD Biosciences, cat. No. 554724) for 4 h following the manufacturer's instructions. The cells and culture supernatants were collected; the supernatants were centrifuged at 500 g for 5 min to eliminate any remaining cells and debris, and stored at -80°C for subsequent cytokine assaying.

Determination of cytokine expression of T cell populations in isolated splenocytes

After *ex vivo* stimulation of splenocytes, as described above, 10^6 cells were fixed and permeabilized with BD Cytofix/Cytoperm™ (BD Biosciences, cat. No. 554714) and labelled with FITC anti–CD4, PE anti–IFNγ and AlexaFluor 647 anti–IL4 antibodies (BD Biosciences, cat. No. 553047, 554412 and 557739, respectively), by following the manufacturer's instructions. Flow cytometry analysis was again performed with a FACS Calibur cytometer using CellQuest Pro software. The populations of cells expressing IFN-γ or IL-10 were calculated as the percentages of cells positive for the corresponding cytokine among the total $CD4^+$ lymphocyte population.

Antibody determination by ELISA

Blood samples extracted after sacrifice were left for 1 h at 4 °C and 1 h at room temperature to allow clotting to occur. After clot formation, the samples were centrifuged at 21,000 g for 15 min to obtain cell-free serum, and stored at -80 °C prior to further analyses. The levels of anti-αSyn IgG1 and IgG2a isotypes and of the total IgG1 and IgG2a antibodies were quantified by following the same procedures as described previously (Labrador-Garrido et al., 2014) except that rat monoclonal anti-mouse IgG1 (Abcam, cat No. ab99603) and rat monoclonal anti-mouse IgG2a (Abcam, cat. No. ab99847), respectively, were used as detection antibodies.

Cytokine determination by ELISA

For quantification of IFN-γ, IL-4, IL-10, and IL-17 levels from culture supernatants or serum, specific ELISA kits, namely mouse IFN-γ and mouse IL-10 BD OptEIATM kits (BD Biosciences), and ELISA Development Kit Murine IL-4 and IL-17 (PeproTech, London, UK), were used according to the manufacturer's instructions.

Experimental design and statistical analyses

Each experimental group consisted of 4-7 mice; the specific numbers (N) are shown in each figure legend. Sample size was estimated using the G*Power 3.1 software (University of Dusseldorf), for one-way ANOVA with a 0.8 power, an alpha value of 0.05 and f value of 3.5; based on the results obtained in our previous studies (Labrador-Garrido et al., 2016; Munoz-Manchado et al., 2016). The data are presented as the mean ±SEM. Statistical analysis was performed by using the IBM SPSS Statistics 20 pack (Chicago, IL, USA) or Sigmastat 3.1 software (Systat Software Inc, San Jose, CA,

USA). In all cases, the statistical differences shown (represented by '*' and '#') are from the different experimental groups compared to the 'vehicle' and the 'MPTP' controls, respectively. A 'P' value of $p<0.05$ was considered to be of statistical significance.

For immunological analysis, the Kruskal-Wallis one-way ANOVA was first performed to evaluate the existence of significant differences among the experimental groups. To determine the differences between groups, and to obtain the P values, the non-parametric Mann-Whitney U test for two independent samples was used.

For the histological and stereological analyses, the data were studied with the one-way ANOVA test with a Bonferroni *post hoc* analysis. In all cases, the normality test (Kolmogorov-Smirnov) and the equal variance test were carried out, and passed through, before applying the ANOVA test. The degrees of freedom and the F values for each ANOVA test are shown in each figure legend.

Results

Adoptive transfer of splenocytes from mice immunized with αSyn/Grp94 leads to αSyn-specific Th1/general immune-regulatory profile in the peripheral system of PD mice.

As carried out previously (Labrador-Garrido et al., 2016), groups of C57BL/6N mice were injected with a pre-incubated mixture of a 1:1 molar ratio of αSyn:Grp94, or with αSyn or Grp94, or the vehicle control. To boost the immune response, the immunogens were applied in the presence of Freund's adjuvant (see Materials and Methods for details). One week after the second (booster) injection, the mice were sacrificed and the spleens extracted for splenocytes isolation.

Isolated splenocytes from mice within each experimental group were pooled and transferred to recipient C57BL/6N healthy mice (see Materials and Methods for details), which were subsequently rendered parkinsonian by the chronic treatment with MPTP that had been characterized recently by our group (Munoz-Manchado et al., 2016). In addition to AT-recipient mice, saline-treated and MPTP-treated non-immunized animals (referred to as saline and MPTP, respectively) served as controls. After completing the MPTP or saline treatment, the mice were evaluated for their locomotive capabilities and, after being sacrificed, their spleen, blood and brain were extracted for immunological, stereological and histological analysis. Interestingly, flow cytometry analysis of the $CD4^+$ T cell population in isolated splenocytes revealed an increase in the ($CD25^+FoxP3^+$) Treg content for mice immunized with αSyn/Grp94 and with Grp94, as compared to non-immunized MPTP-treated mice (13.8±0.4% and 12.4±0.2% vs. 11.1±0.4 %; P=0.011 and P=0.027, respectively) **(Figure 1a)**.

To determine whether αSyn-specific cells in the peripheral system of immunized mice were predominantly immune-regulatory or Th1-type in nature, we measured by ELISA the secreted levels of IL-10 and IFN-γ of isolated splenocytes 24 h after a challenge with αSyn, and calculated the IFN-γ/IL-10 pairwise ratio **(Figure 1b).** The results showed a higher IFN-γ/IL-10 ratio for the αSyn/Grp94-immunized mice than for the MPTP and vehicle controls (1.25±0.06 vs. 0.89±0.07 and 0.81±0.04, respectively; P=0.012 and P=0.003), indicating a Th1-skewing in the αSyn-specific cellular response, and also suggesting that the Treg response promoted by αSyn/Grp94 immunization is not αSyn-specific. In addition, stimulation of isolated splenocytes with LPS resulted in higher IL-10 secretion levels for the αSyn/Grp94 group than for the MPTP and vehicle groups (210±47 pg/mL vs. 83±7 pg/mL and 62±5 pg/mL, respectively; P=0.019 and P=0.004), further pointing to a strengthened antigen-independent immune-regulatory

response in splenocytes resulting from αSyn/Grp94-immunization **(Figure 1c)**. Finally, no differences were detected in the levels of Th17-associated IL-17 secretion in cultured splenocytes stimulated with αSyn **(Figure 1d, left)**. However, unlike in the vehicle and αSyn groups, immunization with αSyn/Grp94 maintained the IL-17 response to LPS at levels comparable to the saline group **(Figure 1d, right)**.

On the other hand, determination of αSyn-specific IgG1 and IgG2a isotypes of serum antibodies by ELISA revealed a significant reduction in the Th2-associated IgG1 isotype content for the αSyn/Grp94 group compared to the MPTP control group (0.019±0.003 AU vs. 0.052± 0.011 AU; P=0.011) **(Figure 1e)**. Interestingly, this effect was coupled to an increase in the IgG1 isotype content of total IgG antibodies (42±3.1 AU vs. 19±1.9 AU; P=0.020) and reflected by an increase trend in the IgG1/IgG2a pairwise ratio, compared to the vehicle group (13.5±2.7 vs. 5.9±2.7; P=0.088) **(Figure 1f)**. Taken together, these results point to a Th1-skewing in the αSyn-specific response in addition to an immuno-regulatory/Th-2 general phenotype, at the cellular and humoral levels, in mice vaccinated with Grp94-chaperoned αSyn via AT of splenocytes, after the experimental induction of parkinsonism.

Suppression of microgliosis in the chronic PD model by adoptive transfer of splenocytes from αSyn/Grp94-immunized mice.

The development of sustained microgliosis is a well-established neuropathological feature of PD that has recently been well associated to the neurodegenerative process (McGeer et al., 1988; Hirsch and Hunot, 2009; Fakhoury, 2016). Indeed, we have previously shown a permanent neuroinflamatory reaction, including general microglial activation, in the same chronic MPTP parkinsonian mouse model used in this study (Munoz-Manchado et al., 2016). We therefore analyzed whether the immunological

conversion induced by αSyn/Grp94 AT vaccination could possibly modify the chronic neuroinflamatory response associated with experimental PD. As expected, the chronic MPTP treatment induced a significant increase of activated $Iba1^+$ microglial cells in the *substantia nigra pars compacta* (SNpc) and *striatum* **(Figure 2** and **Table 1)**. Remarkably, AT vaccination with αSyn/Grp94 uniquely produced a significant reduction of activated $Iba1^+$ cells in the SNpc, compared to the MPTP control group and vehicle-injected mice **(Figure 2a,b** and **Table 1)**. In addition, AT vaccination with Grp94 alone and especially, αSyn/Grp94, showed a reduction in activated Iba1+ cells in *striatum* **(Figure 2a,c** and **Table 1)**. Examination of astrogliosis levels associated with the PD model (Muñoz-Manchado et al., 2016), on the other hand, showed a reduction trend as a result of AT vaccination with both Grp94 alone and αSyn/Grp94, compared to MPTP or vehicle control mice **(Supplem. Fig. 1**). Finally, we also analyzed the levels of protection from both MPTP-induced TH^+ neuronal loss in the SNpc and parkinsonian motor symptoms for these groups. We did not observe statistical significance compared to MPTP-vehicle AT-vaccinated controls (data not shown), which was not surprising given that dopaminergic neuronal cell death in our model is known to be primarily caused by MPTP itself (Bezard and Przedborski, 2011).

In conclusion, our results showed that AT of splenocytes from mice immunized with αSyn/Grp94 diminished the neuroinflammatory reaction associated with experimental PD, specifically by producing a significant suppresion of microglial activation, the process that is considered to be the main mediator of neuroinflammation (Fakhoury, 2016).

Direct immunization with αSyn/Grp94 supports an αSyn-specific Th1/general Th2-immune-regulatory profile in the peripheral system of PD mice.

Having observed that αSyn/Grp94 vaccination by AT of splenocytes suppressed the development of microgliosis, we sought to test our immunization strategy using a direct immunization (DI) procedure. After immunizing C57BL/6N healthy mice (with either αSyn, Grp94, or αSyn/Grp94 preparations, or with vehicle) as previously described, the animals were subjected to a subdose of MPTP (or saline) administration for three months. Apart from the saline-treated (saline) and MPTP-treated (MPTP) non-immunized mouse groups, which served as controls, one saline-treated group was immunized with αSyn/Grp94 to test the effect of this treatment in the absence of MPTP-induced pathology.

After the end of the MPTP (or saline) treatment, mice were tested for their locomotive capabilities and after sacrifice; their spleen, blood and brain were extracted for immunological and histological analyses. In this case, flow cytometry studies of splenocytes isolated from immunized mice showed no significant changes in the ($CD4^+CD25^+Foxp3^+$) Treg cell content between the different groups **(Figure 3a)**. However, flow cytometry analysis of isolated splenocytes for IFN-γ- and IL-4-expression revealed an increase trend in the ratio of IFN-γ-expressing cells after a challenge with αSyn in the αSyn/Grp94-immunized group compared to the MPTP and vehicle controls **(Figure 3b)**. Conversely, a reduction in this ratio was observed for the αSyn/Grp94 group after stimulation with anti-CD3/anti-CD28 **(Figure 3b)**.

In order to further characterize the cellular immunity in vaccinated mice, we cultured splenocytes isolated from immunized mice and measured IFN-γ, IL-10 and IL-17 secreted levels in culture supernatants, by ELISA. The analysis after challenge with αSyn again revealed an increase in the ratio of IFN-γ/IL-10 secretion levels in the

αSyn/Grp94 group compared to the MPTP group (51.5±3.2 vs. 15.3±3.7; P=0.047) **(Figure 3c)**. When compared to the vehicle group (6.2±1.4), the αSyn (25.5±9.5), Grp94 (22.7±6.4) and, especially, the αSyn/Grp94 (51.5±3.2), groups showed significant increments in this ratio (P=0.016, 0.016, and 0.009, respectively), further indicating a skewing towards a Th1 response to αSyn stimulation in αSyn/Grp94-immunized mice. Interestingly, this skewing was accompanied by an increase in IL-17 secreted levels in the αSyn/Grp94 group (27.9±3.7 pg/mL) compared to the MPTP mice (14.6±1.7; P=0.076) and to the vehicle group (12.4±2.1 pg/mL; P=0.012), followed by the αSyn (23.4±3.8 pg/mL; P=0.010) and Grp94 (23.1±2.9 pg/mL; P=0.012) immunized mice **(Figure 3d, left)**. Given that IL-17 is involved in the Th17-mediated autoimmune response, we tested whether or not such an increase could be also measured after polyclonal stimulation of cultured splenocytes with anti-CD3/anti-CD28. Strikingly, our results showed a complete lack of IL-17 response in the αSyn/Grp94 group (174±58 pg/mL), that was comparable to the effect in MPTP mice (172±36 pg/mL), in contrast to splenocytes from the αSyn (782±104 pg/mL; P=0.014) and Grp94 (517±96 pg/mL; P=0.016) groups, which induced elevated IL-17 levels **(Figure 3d, right),** suggesting that the IL-17 response resulting from αSyn/Grp94 immunization in the PD model is constrained to αSyn-specific stimulation.

We next quantified the levels of anti-αSyn IgG1 and IgG2a isotypes in serum antibodies by ELISA and observed an increase in the IgG1 content in mice immunized with αSyn/Grp94 (0.50±0.14 AU) followed by Grp94 (0.162±0.002 AU), compared to MPTP (0.045±0.009 AU; P=0.009 for both) and vehicle (0.08±0.011 AU; P=0.006 for both) control groups **(Figure 3e)**; even stronger increments in IgG2a levels were seen only in the αSyn/Grp94 group (2.2±0.51 AU) compared to the MPTP (0.93±0.02 AU;

P=0.009) and vehicle (0.13±0.02 AU; P=0.006) groups **(Figure 3e)**. This 10-25-fold increment in the levels of αSyn-specific antibodies clearly reveals a differential feature of αSyn/Grp94 immunization via DI, as opposed to that observed for the AT procedure **(Figure 1e)**. Moreover, the analyzed pairwise ratios of anti-αSyn IgG1/IgG2a antibodies revealed a significant increase for the αSyn group (1.6±0.4; P=0.028) and a significant reduction for the αSyn/Grp94 group (0.24±0.04; P=0.009) compared to the MPTP group (0.51±0.05), indicating a skewing of the αSyn-specific immunity towards a Th2 or Th1 profile, respectively **(Figure 3e, inset)**. By contrast, the analyzed IgG1/IgG2a isotype ratio for total (i.e. not antigen-specific) IgG antibodies in serum showed an increase trend for the αSyn/Grp94 group, especially compared to the vehicle, αSyn and Grp94 groups **(Figure 3f)**, further supporting a Th2-type general phenotype induced by the combination of proteins. Taken together, these results point to a Th1/Th17-shift in the αSyn-specific response and a Th2-skewed general phenotype, at the cellular and humoral levels, in mice vaccinated by direct immunization with αSyn/Grp94.

Direct immunization with αSyn/Grp94 suppresses microgliosis associated with the chronic PD experimental model.

As discussed above for the animals subjected to AT immunization, we analyzed the degree of microgliosis of the different experimental groups subjected to DI. Remarkably, and similarly to the case of AT, mice directly immunized with αSyn/Grp94 that were subjected to MPTP treatment showed a clear suppression in the number of activated $Iba1^+$ cells compared to both MPTP controls and vehicle-injected animals in both the SNpc and *striatum* **(Figure 4** and **Table 2)**. Of note, a decrease trend in microglial activation in *striatum* was also observed for the Grp94 mice,

although it did not reach statistical significance compared to vehicle controls. Remarkably, no significant differences in the number of activated microglial cells were found between saline controls and αSyn/Grp94-saline treated mice **(Figure 4b,c** and **Table 2)**, indicating that the main effect of the αSyn/Grp94 immunization on microglial cells is a selective blockage of microglia activation occurring under conditions of experimental parkinsonism. Finally, no essential differences between experimental groups and vehicle-injected controls were observed in the analyses of astrogliosis levels, SNpc TH^+ cells quantification, or motor tests (data not shown), which could be explained by a possible masking effect of the adjuvant on TH^+ cells in the MPTP mouse model, as previously described (Yong et al., 2011).

In conclusion, the data obtained both from the AT and DI immunization strategies show that αSyn/Grp94 immunization promotes a switch of the peripheral immune profile associated with experimental parkinsonism toward a Th1 αSyn-specific immunity together with a general immune-regulatory/Th2 phenotype. This is the first report of an αSyn-targeting vaccination approach leading to suppression of microgliosis in the CNS, in the context of PD.

Discussion

In the last decade, passive and active immunotherapy has emerged as an attractive and powerful approach to developing therapies for PD and other synucleinopathies (Olson and Gendelman, 2016; Schneeberger et al., 2016). Initial evidence for the neuroprotective potential of αSyn-based vaccination was provided by a study aimed to produce a strong αSyn-specific antibody response by immunizing mice with full-length αSyn protein, in an αSyn-overexpressing model (Masliah et al., 2005).

The importance of specific T-cell subsets for PD-associated neurodegeneration, and particularly of the neuroprotective role of the $CD4^+CD25^+$ population, was later demonstrated in a study by using AT of *in vitro*-activated T cells from donor mice to MPTP-treated recipient mice (Reynolds et al., 2007). Furthermore, treatment with neuropeptide-induced ($CD4^+CD25^+$) Treg cells was shown to attenuate the neurotoxicity caused by *in vitro*-Th17 polarized $CD4^+$ cells isolated from mice immunized with the pathological form of αSyn N-4YSyn, suggesting a detrimental role of the Th17 population in this context (Reynolds et al., 2010). More recently, immunization with αSyn has been shown to reduce the level of αSyn aggregates and to promote the recruitment into the CNS of Treg cells and αSyn-specific antibodies in a rat model of PD (Sanchez-Guajardo et al., 2013). These studies indicate that the right subset of T cells must be engaged and the appropriate αSyn epitopes targeted, in the design of effective immunotherapeutic approaches against PD. Furthermore, they indicate that αSyn-targeted immunotherapies may have the potential to attenuate neurodegeneration-linked neuroinflammation (Olson and Gendelman, 2016).

Other strategies for immunotherapy have been based on the production of high levels of αSyn-specific antibodies resulting from the immunization of mice with αSyn-derived peptides, to clear αSyn aggregates (Ghochikyan et al., 2014; Mandler et al., 2014). These results from studies performed in αSyn-based PD models have demonstrated that generating an αSyn-specific humoral response may be beneficial in the context of αSyn-overexpression (Mandler et al., 2014). Nevertheless, despite several active immunization strategies proposed, no pre-clinical vaccination study targeting α-synuclein had thus far shown to abrogate the progressive microglial activation associated to PD pathology, as shown in the present work. Indeed, it is currently thought that alternative active vaccination approaches with disease-modifying potential

are needed to enter the development pipeline towards the clinic (Schneeberger et al., 2016), especially those candidates aiming at both targeting pathological αSyn and harnessing the neuroinflammatory process (Romero-Ramos et al., 2014).

For more than two decades, HSP-peptide complexes extracted from tumors have been studied intensively as cancer vaccines, and been found to be safe and effective against a range of tumors (Murshid et al., 2011). For example, Grp94, Hsp70, Hsp110 and Grp170 have been shown to produce anti-tumor effects in a variety of animal models (Udono and Srivastava, 1993, 1994; Wang et al., 2001) and the use of exogenously administered chaperones in immunotherapeutic approaches has also been explored for the treatment of autoimmune diseases (Binder, 2014).

We have recently shown that αSyn chaperoned with certain HSPs elicits a differential immune response *in vitro* and *in vivo* (Labrador-Garrido et al., 2014; Labrador-Garrido et al., 2016). In the present study, we have investigated, for the first time, the impact of immunization with HSP-chaperoned αSyn on the course of a neurodegenerative misfolding disease such as PD, by characterizing the response elicited in the peripheral system and its possible effects at the CNS level.

We studied two different vaccination procedures, the AT of splenocytes from immunized mice and the DI approach, to render the mice parkinsonian by chronic administration of low doses of MPTP (Munoz-Manchado et al., 2016). We chose the MPTP model as it has been shown to reproduce, among others, several immunological features of PD pathology, including altered T cell subsets and sustained microgliosis (Bezard and Przedborski, 2011; Tieu, 2011). This widely used pathological model of PD is considered especially suitable for studying non-cell autonomous mechanisms of neurodegeneration, including the role of glial cells and neuroinflammation in the onset and development of the disease (Bezard and Przedborski, 2011; Dehay et al., 2016).

Our results show that both the strategies based on αSyn/Grp94 immunization resulted in a common immunologic phenotype in the periphery, involving both the cellular and humoral arms of the immune system. Such αSyn/Grp94-supported immune profile is characterized by an αSyn-specific Th1-skewed profile, accompanied by a shift toward immune-regulatory/Th2-type general phenotype **(Figure 5)**. Of note, the essentially unaltered Treg cell content in DI-immunized mice indicates that other cell populations are engaged in suppression of microgliosis in αSyn/Grp94-immunized mice. In addition, both vaccination approaches —which required only two or three injections— proved to have long-lasting effects and to result in strong suppression of pathology-associated microgliosis.

The distinct reshaping of the peripheral immunity resulting from αSyn/Grp94 vaccination, as well as its unanticipated protective effects on neuroinflammation proved to be pathology-dependent, supporting the view that these long-term effects are the outcome of both vaccine-induced immunity and the initiation and development of the pathological processes. Such protective immune profile might still be reinforced or tailored by designing an αSyn/Grp94 vaccination protocol involving the ligand-induced activation of the key T-cell functional regulator aryl hydrocarbon receptor (AhR) (Quintana, 2013).

Based on the present study, we propose that prophylactic or early αSyn/Grp94 immunization allows immunity to be harnessed towards a specific Th1 αSyn-directed response that could be protective in the context of a chronic and slow buildup of PD pathology. This response would support the rapid and efficient elimination of toxic elements derived from αSyn-linked necrotic cells at the initial stages of disease, limiting damage and the subsequent recruitment of further pro-inflammatory effects that are likely to be overwhelming. In such context, the αSyn-targeting component of the

αSyn/Grp94-supported response could be expected to confer additional protection. This phenomenon, added to a general state of immune-regulation (Treg/Th2) would enable the response to be high under control and easily down-regulated once the damage has ceased. Interestingly, boosting T cell-mediated autoimmunity through vaccination with modified self-epitopes against tumor and autoimmune disease (Cohen, 2014), or with 'attenuated' CNS self-antigens to counteract chronic neuroinflammation, have been found to promote beneficial immune responses —including Th1-skewed responses— in models of acute nerve injury and motor neuron disease (Shaked et al., 2005; Kunis et al., 2013; Raposo et al., 2014). Additionally, given that HSPs have been shown to alter the migratory capacity of immune cells (Binder et al., 2000; Nussbaum et al., 2006; Simard et al., 2011; Dieterich et al., 2013), it might also be possible that T cell chemotaxis is being differentially modulated in αSyn/Grp94-vaccinated mice, therefore affecting their ability to interact with CNS-resident cells.

The present study is the first that has utilized active αSyn-based vaccination to show suppression of the persistent microgliosis state developed during the course of PD pathology. We propose that the distinct and complementary Th1/immune-regulatory immunological profile generated by vaccination with αSyn/Grp94 enables the specific targeting of αSyn and the prevention of further spreading of pathological foci, while maintaining immune homeostasis and avoiding neuroinflammation in the long-term. Based on this model, such a vaccination strategy would serve as a novel and potentially powerful immunotherapeutic approach for PD and other synucleinopathies. Although in this study we have focused on a protective vaccination strategy, where the vaccination is administered prior to the MPTP-induced damage, it would be highly attractive to test by further studies the potential use of αSyn/Grp94 vaccination following the MPTP treatment to revert an existing neuroinflammatory state.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Immune response characterization in AT recipient chronic MPTP-treated parkinsonian mice. a. Determination of the Treg ($CD4^+$ $CD25^+$ $Foxp3^+$) cell population from the $CD4^+$ lymphocytes of isolated splenocytes (saline, n=4; MPTP, n=4; vehicle, n=5; αSyn, n=5; Grp94, n=6; αSyn/Grp94, n=6). Representative images from the flow cytometry analysis are shown on the right. **b.** IL-10 levels in the supernatants of cultured splenocytes from recipient mice, 24 h after treatment with LPS (0.5 mg/mL), determined by ELISA (saline, n=4; MPTP, n=5; vehicle, n=5; αSyn, n=6; Grp94, n=5; αSyn/Grp94, n=7). **c.** IFN-γ and IL-10 levels, measured by ELISA, in the supernatants of cultured splenocytes from immunized mice, 24 h after *in vitro* treatment with αSyn (20 μg/mL); the pairwise IFN-γ/IL-10 ratios were calculated by dividing the measured IFN-γ cytokine level by the measured IL-10 cytokine level for each mouse (saline, n=4; MPTP, n=4; vehicle, n=6; αSyn, n=6; Grp94, n=5; αSyn/Grp94, n=6). **d.** IL-17 levels in the supernatants of splenocytes isolated from AT-vaccinated mice, 24 h after challenge with αSyn (20 μg/ml, left) or LPS (1 μg/mL, right), measured by ELISA (saline, n=4; MPTP, n=5; vehicle, n=5; αSyn, n=6; Grp94,n=5; αSyn/Grp94, n=7). **e.** The anti-αSyn IgG1 or IgG2a isotype contents in serum were calculated by dividing the levels of anti-αSyn IgG1 or anti-αSyn IgG2a antibodies by the total IgG levels, obtained by ELISA measurement, for each mouse (saline, n=4; MPTP, n=5; vehicle, n=6; αSyn, n=6; Grp94, n=6; αSyn/Grp94, n=7). **f.** Total IgG1 and IgG2a antibody levels in serum, determined by ELISA. Pairwise IgG1/IgG2a ratios were calculated by dividing the measured IgG1 level by the measured IgG2a level for each mouse (saline, n=4; MPTP, n=5; vehicle, n=5; αSyn, n=6; Grp94, n=6; αSyn/Grp94, n=7). AU: arbitrary units. Values are expressed as mean ±S.E.M and statistical analysis was performed using the Mann-Whitney test. *$p<0.05$; **$p<0.01$; ***$p<0.001$ relative to the

vehicle group. $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ relative to non-immunized MPTP controls.

Figure 2. Suppression of microglial activation by αSyn/Grp94 AT vaccination in chronic MPTP-treated parkinsonian mice. a. Representative high magnification SNpc and striatal images after Iba1 immunohistochemistry, from saline, MPTP-treated mice, and AT-vaccinated mice (vehicle, αSyn, Grp94 and αSyn/Grp94) subjected to MPTP treatment. Note the presence of activated microglia in the MPTP-treated mice and the clear reduction produced by the αSyn/Grp94 AT. **b, c.** Stereological quantification of activated $Iba1^{+}$ microglial cell density in the SNpc (b) and *striatum* (c) of the same experimental groups described in A. (saline, n=4; MPTP, n=4; vehicle, n=6; αSyn, n=6; Grp94, n=6; αSyn/Grp94, n=6). Values are expressed as mean ±S.E.M and the statistical analysis was performed with the one-way ANOVA test with a Bonferroni *post hoc* analysis. *p<0.05; **p<0.01; **p<0.001 relative to the vehicle group. $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ relative to non-immunized MPTP controls. The degree of freedom (DF) and F values of the ANOVA test were as follows: B, 5 and 11.840; D, 5 and 18.523.

Figure 3. Immune response characterization in DI vaccinated MPTP-treated mice. a. Determination of the Treg ($CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$) cell population among the $CD4^{+}$ lymphocytes of isolated splenocytes (saline, n=4; saline αSyn/Grp94, n=4; MPTP, n=5; vehicle, n=5; αSyn, n=5; Grp94, n=5; αSyn/Grp94, n=5). **b.** Pairwise Th1/Th2 cell ratios in splenocytes isolated from immunized mice after αSyn or anti-CD3 antibody challenge, analyzed by flow cytometry. Th1 and Th2 cell populations were calculated as the IFN-γ or IL-4 producing cells, respectively, within the $CD4^{+}$ lymphocyte

population. The splenocytes were collected and stained, 24 h after treatment with α-synuclein (20 μg/mL) or cultured for 24 h in anti-CD3 and anti-CD28 coated plates. Pairwise Th1/Th2 ratios were calculated by dividing the measured $CD4^+$ IFN-γ^+ cells by the measured $CD4^+$ IL-4^+ cells for each mouse (saline, n=4; saline αSyn/Grp94, n=4; MPTP, n=5; vehicle, n=5; αSyn, n=5; Grp94, n=5; αSyn/Grp94, n=5). **c.** IFN-γ and IL-10 levels were measured by ELISA in the supernatants of cultured splenocytes from immunized mice, 24 h after *in vitro* treatment with αSyn (20 μg/mL) and the pairwise IFN-γ/IL-10 ratios were calculated for each mouse (n=5 for all groups). **d.** IL-17 levels in the supernatants of cultured splenocytes from immunized mice, 24 h after treatment with αSyn (20 μg/ml, left) or anti-CD3/anti-CD28 (right), measured by ELISA (n=5 for all groups). **e.** The anti-αSyn IgG1 or IgG2a isotype contents in serum were calculated by dividing the levels of anti-αSyn IgG1 or anti-αSyn IgG2a antibodies by the total IgG levels, obtained by ELISA measurement, for each mouse (n=5 for all groups). **f.** The total IgG1/IgG2a pairwise ratios were calculated by dividing the total IgG1 levels by the total IgG2a levels for each mouse, as measured by ELISA (n=5 for all groups). AU: arbitrary units. Values are expressed as mean ±SE.M and the statistical analysis was performed using the Mann-Whitney test. *p<0.05; **p<0.01; ***p<0.001 relative to the vehicle group. $^{\#}$p<0.05; $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001, relative to non-immunized MPTP controls.

Figure 4. Suppression of microglial activation by αSyn/Grp94 DI vaccination in chronic MPTP-treated parkinsonian mice. a. Representative high magnification SNpc and striatal images after Iba1 staining, from saline, MPTP-treated mice, and DI vaccinated mice (vehicle, αSyn, Grp94 and αSyn/Grp94) subjected to chronic MPTP treatment. Note the presence of activated microglia on the MPTP-treated mice and the

clear reduction resulting in particular from the αSyn/Grp94 DI vaccination. **b, c.** Stereological quantification of $Iba1^+$ microglial cell density in SNpc (b) and striatum (c) of saline (n=4), saline αSyn/Grp94 DI (n=4); and the MPTP-treated mice: MPTP controls (n=4); vehicle, n=6; αSyn, n=6; Grp94, n=5; and αSyn/Grp94, n=5). Values are expressed as mean ±S.E.M and the statistical analysis was performed with the one-way ANOVA test with a Bonferroni *post hoc* analysis. *p<0.05; **p<0.01; ***p<0.001 relative to the vehicle group. $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001 relative to non-immunized MPTP controls. The degree of freedom (DF) and F values of the ANOVA test were as follows: B, 6 and 10.209; D, 6 and 14.357.

Figure 5. Vaccination with αSyn/Grp94 supports a mixed Th1/immune-regulatory response resulting in long-term CNS protection in a chronic model of PD. AT- and DI-αSyn/Grp94 vaccinated mice developed a common immune profile in the peripheral system, consisting of an αSyn-specific Th1-type response coupled with a general Th2-skewed immune-regulatory response, in a progressive chronic model of PD. Importantly, the elicited immune profile involved both the cellular and humoral arms of immunity. By the end-stage of PD modeling, both αSyn–based vaccination strategies resulted in strong suppression of PD-associated microgliosis.

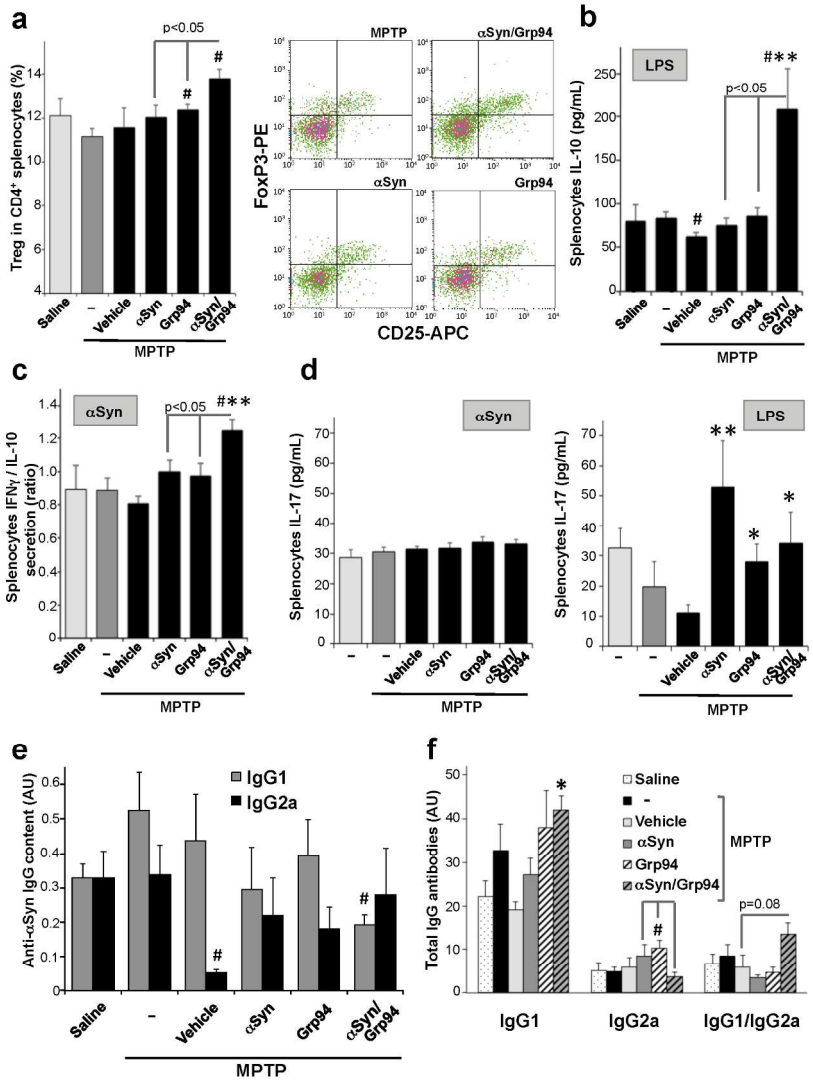

Figure 1

Figure 1. Immune response characterization in AT recipient chronic MPTP-treated parkinsonian mice.

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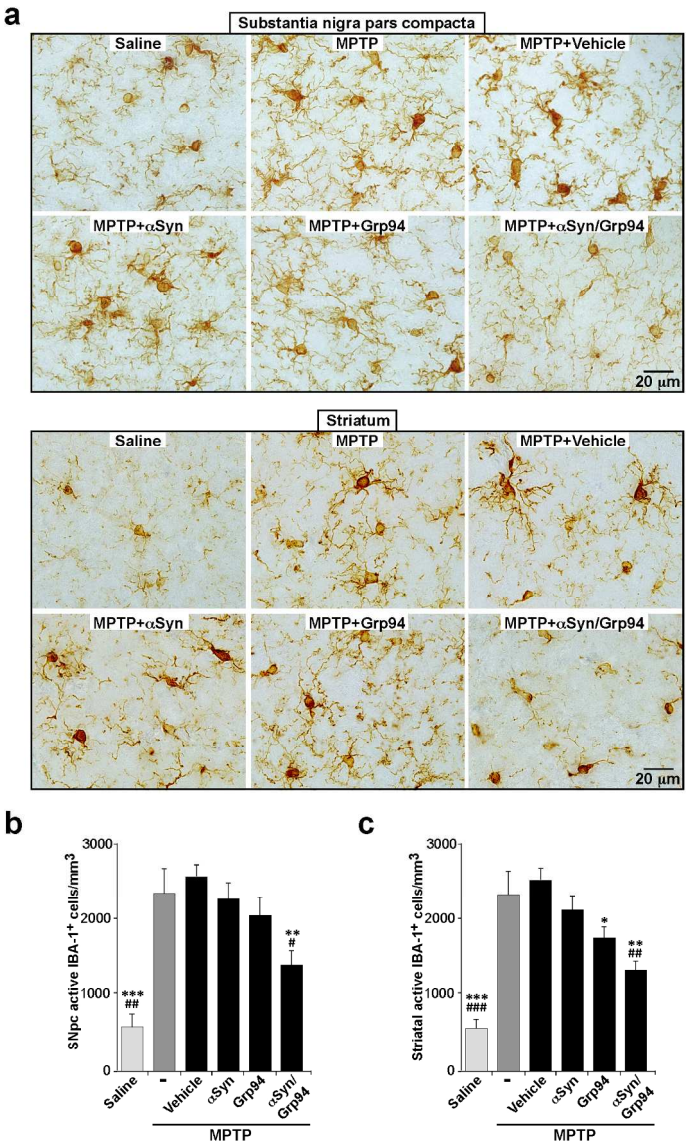

Figure 2

Figure 2. Suppression of microglial activation by αSyn/Grp94 AT vaccination in chronic MPTP-treated parkinsonian mice.

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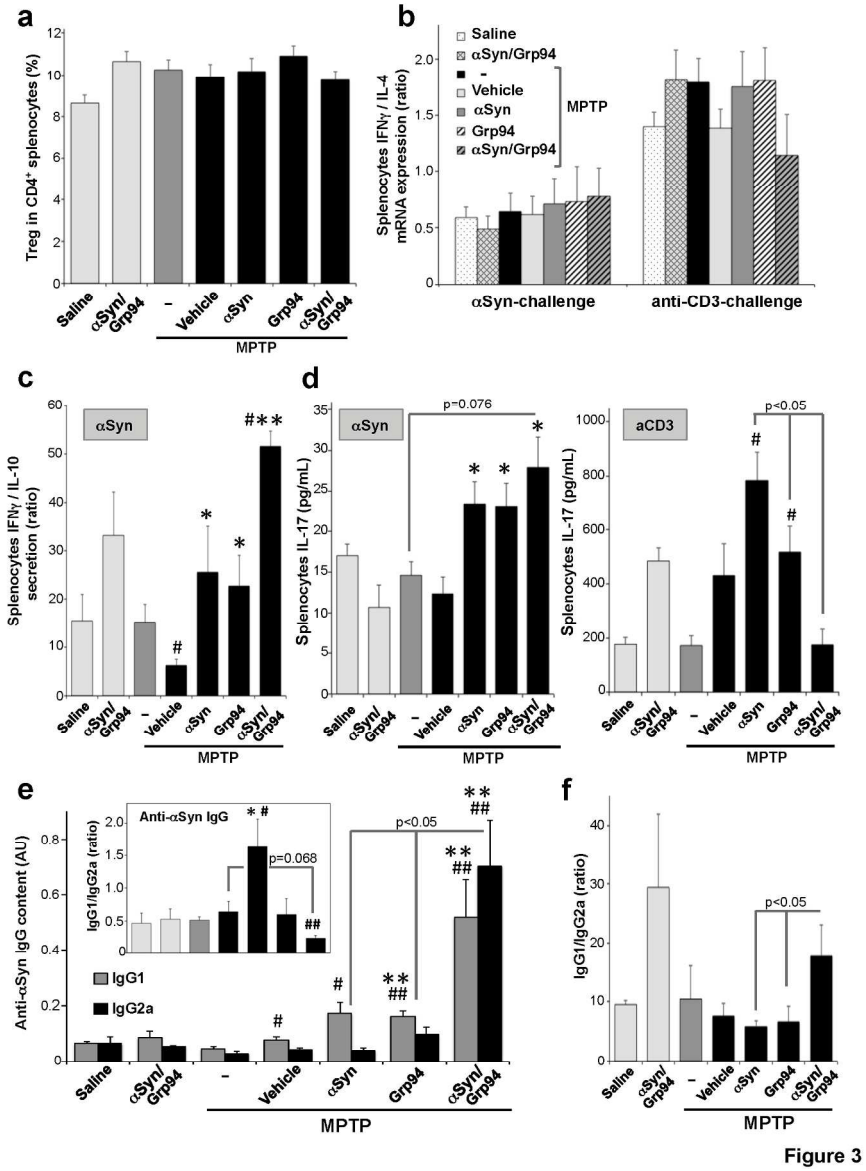

Figure 3

Figure 3. Immune response characterization in DI vaccinated MPTP-treated mice.

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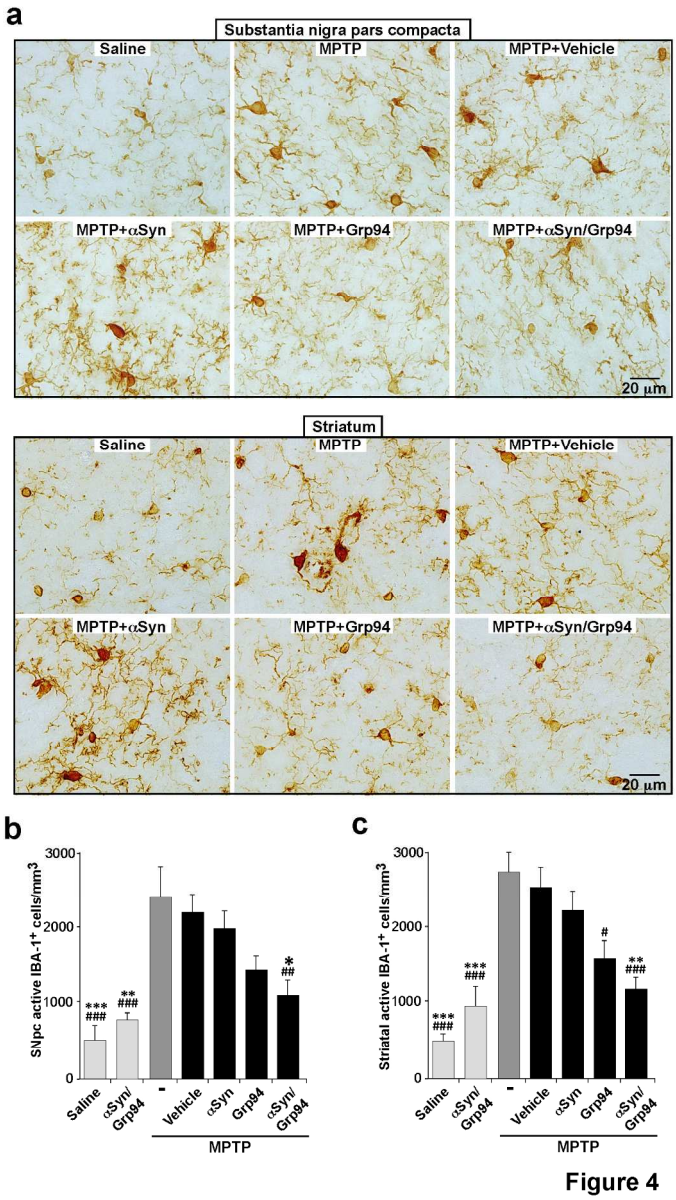

Figure 4

Figure 4. Suppression of microglial activation by αSyn/Grp94 DI vaccination in chronic MPTP-treated parkinsonian mice.

136x245mm (300 x 300 DPI)

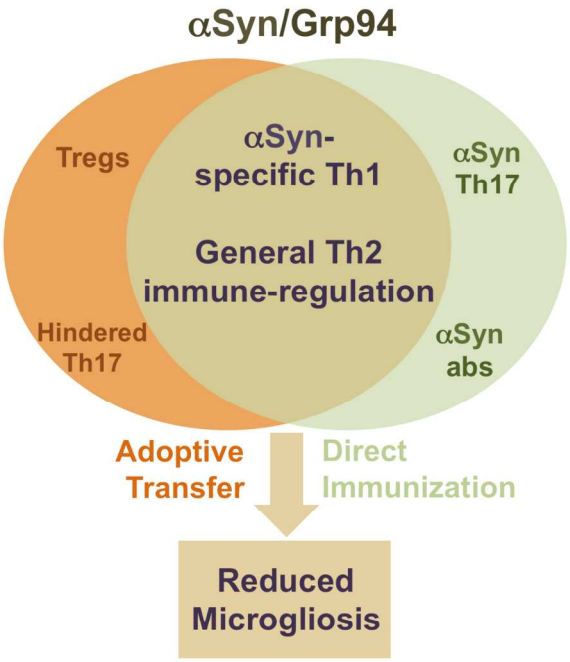

Figure 5

Figure 5. Vaccination with αSyn/Grp94 supports a mixed Th1/immune-regulatory response resulting in long-term CNS protection in a chronic model of PD.

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Table 1. Stereological quantification of the total, resting and activated (Iba1+) microglial cell density in the SNpc and striatum from AT-vaccinated animals and controls.

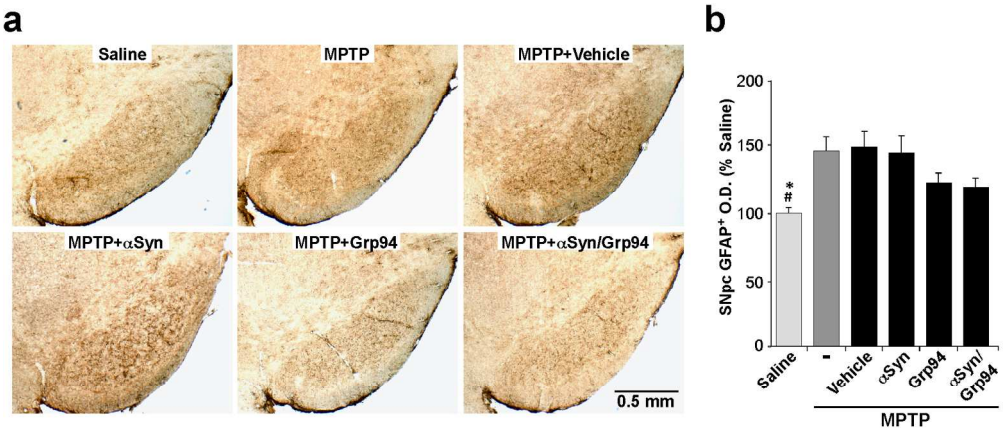
Substantia nigra pars compacta				
Experimental Group	**N**	**Total Iba1$^+$ cells**	**Resting Iba1$^+$ cells**	**Activated Iba1$^+$ cells**
Saline	4	13952 ± 1069	13338 ± 1063$^{\#,***}$	615± 164$^{\#\#,***}$
MPTP	4	12845 ± 209	10498 ± 252	2351 ± 221
MPTP + Vehicle	6	12258 ± 363	9726± 265	2533 ± 138
MPTP + αSyn	6	12086 ± 299	9844 ± 291	2243 ± 205
MPTP + Grp94	6	12458 ± 356	10416 ± 428	2042 ± 235
MPTP + αSyn/Grp94	6	12035 ± 613	10641± 579	1394 ± 178$^{\#,**}$
Striatum				
Experimental Group	**N**	**Total Iba1$^+$ cells**	**Resting Iba1$^+$ cells**	**Activated Iba1$^+$ cells**
Saline	4	15772 ± 884	15248 ± 876	524 ± 70$^{\#\#\#,****}$
MPTP	4	15230 ± 884	12984 ± 998	2245 ± 239
MPTP + Vehicle	6	15849 ± 486	13346 ± 513	2502 ± 160
MPTP + αSyn	6	15082 ± 401	12975 ± 235	2107 ± 187
MPTP + Grp94	6	14582 ± 613	12828 ± 545	1754 ± 151*
MPTP + αSyn/Grp94	6	15343 ± 241	14005 ± 195	1337 ± 75$^{\#\#,**}$

Values are expressed as mean ±S.E.M and the statistical analysis was performed with the one-way ANOVA test with a Bonferroni post hoc analysis. *p< 0.05; **p<0.01; ***p<0.001 respect to the vehicle group. #p<0.05; ##p<0.01; ###p<0.001 respect to non-immunized MPTP controls. The degree of freedom (DF) and F values of the ANOVA test were as follows: SNpc-total microglia, 5 and 1.676; SNpc-rest microglia, 5 and 5.761; SNpc-active microglia, 5 and 11.840; striatum-total microglia, 5 and 0.727; striatum-rest microglia, 5 and 2.466; striatum-active microglia, 5 and 18.523.

Table 2. Stereological quantification of the total, resting and activated (Iba1+) microglial cell density in the SNpc and striatum from DI-vaccinated animals and controls.

Substantia nigra pars compacta				
Experimental Group	**N**	**Total Iba1$^+$ cells**	**Resting Iba1$^+$ cells**	**Activated Iba1$^+$ cells**
Saline	4	12567± 1040	12055 ± 1091	513± 193$^{###,***}$
Saline + αSyn/Grp94	4	11214 ± 628	10441 ± 683	774± 72$^{###,***}$
MPTP	4	11534± 709	9045± 847	2489± 363
MPTP + Vehicle	6	12352± 530	10192± 604	2159± 230
MPTP + αSyn	6	12083 ± 355	10134 ± 504	1948± 225
MPTP + Grp94	5	11480 ± 488	10070 ± 442	1410± 168
MPTP + αSyn/Grp94	5	11383 ± 297	10293 ± 185	1090± 183$^{##,*}$
Striatum				
Experimental Group	**N**	**Total Iba1$^+$ cells**	**Resting Iba1$^+$ cells**	**Activated Iba1$^+$ cells**
Saline	4	14757± 535	14269 ± 514	487 ± 85$^{###,***}$
Saline + αSyn/Grp94	4	15003 ± 744	14036 ± 647	967 ± 266$^{###,***}$
MPTP	4	14762 ± 927	11859 ± 990	2904 ± 225
MPTP + Vehicle	6	15037 ± 524	12406 ± 607	2632 ± 263
MPTP + αSyn	6	14739 ± 562	12531 ± 727	2209 ± 251
MPTP + Grp94	5	13637 ± 724	12066 ± 811	1571 ± 220$^{#}$
MPTP + αSyn/Grp94	5	13877 ± 490	12710 ± 351	1167 ± 141$^{##,**}$

Values are expressed as mean ±S.E.M and the statistical analysis was performed with the one-way ANOVA test with a Bonferroni post hoc analysis. *p< 0.05; **p<0.01; ***p<0.001 respect to the vehicle group. #p<0.05; ##p<0.01; ###p<0.001 respect to non-immunized MPTP controls. The degree of freedom (DF) and F values of the ANOVA test were as follows: SNpc-total microglia, 6 and 0.812; SNpc-rest microglia, 6 and 1.673; SNpc-active microglia, 6 and 10.209; striatum-total microglia, 6 and 0.780; striatum-rest microglia, 6 and 1.609; striatum-active microglia, 6 and 14.357.



Supplementary Figure 1. a. Representative coronal sections of mesencephalon, showing the SNpc after GFAP immunostaining from saline, MPTP-treated mice, and AT-vaccinated mice (vehicle, αSyn, Grp94 and αSyn/Grp94) subjected to MPTP treatment. b. Quantification of the astroglial reaction measured by optical density (OD) after GFAP immunostaining detected on the SNpc of the same experimental groups described in 'a' (saline, n=4; MPTP, n=5; vehicle, n=6; ±Syn, n=6; Grp94, n=6; ±Syn/Grp94, n=6). Values are expressed as mean ± S.E.M and the statistical analysis was performed with the one-way ANOVA test with a Bonferroni post hoc analysis. *p<0.05 relative to the vehicle group. #p<0.05; relative to non-immunized MPTP controls. The degree of freedom (DF) and F values of the ANOVA test were 5 and 3.723.

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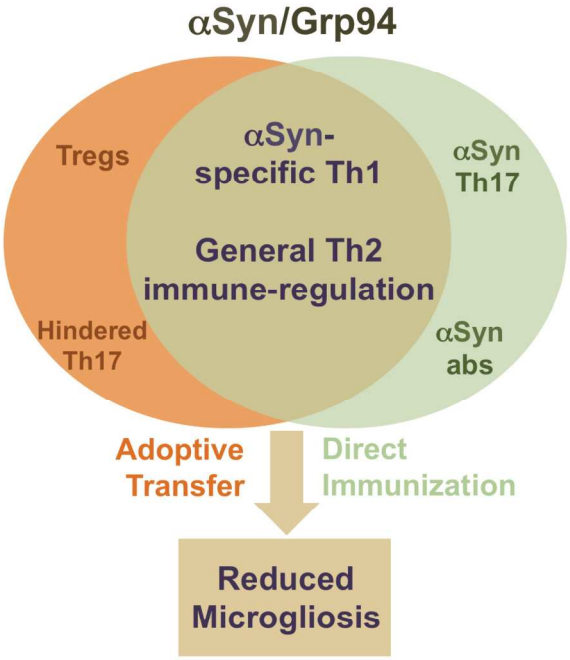

Figure 5

For TOCI purposes

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